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DEVELOPMENT OF A SENSITIVE DNA ASSAY FOR THE AIDS VIRUS, HTLV-III/LAV

FINAL REPORT

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The study was undertaken in order to devise a quantitative assay of human immunodeficiency virus type 1 (HIV-1) load in patients' tissues. No methods are currently available which are both sensitive and specific for this purpose. Our method utilizes blood mononuclear cell or tissue DNA. HIV-1 sequences are amplified by the primer chain amplification reaction (PCR) technique. The reaction products are detected and quantitated by polyacrylamide or agarose gel electrophoresis, ethidium bromide stain and densitometry. This method has proven successful using a single set of primers in detection of HIV-1 DNA sequences from 10 or 12 HIV-1 infected individuals, and 0 of 10 uninfected individuals. Methods of quantitation using standard curves with defined amounts of HIV-1 DNA sequences and internal controls have been developed. Preliminary experiments are reported applying this method to quantitation of HIV sequences in more than 50 patients. In addition, we have developed a sensitive and specific assay for HTLV-I and HTLV-II DNA sequences using the same methodology and we have successfully, applied it to the evaluation of blood samples from asymptomatic HTLV-I infected individuals and individuals with adult T cell leukemia-lymphoma

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Foreword

1) Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

2) For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

3) The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

Background and Significance

HIV-1 infection may cause no symptoms, an acute influenza-like illness, lymphadenopathy syndrome, acquired immunodeficiency disease syndrome (AIDS)-related complex (ARC) (including night sweats, fever, diarrhea, weight loss, oral candidiasis), or AIDS (including neurological disease, opportunistic infections, or malignancies) (1). A significant number of infected individuals will progress from asymptomatic to symptomatic stages of disease (2). There is considerable data to suggest that virus load and immunosuppression is a major determinant of disease stage (3,4). Immunosuppression is likely directly related to virus load as suggested by both in vitro studies as well as clinical studies (4,5). Thus measurements of virus load are likely to be critical for prognostic determinations.

There are currently no sensitive and specific methods of estimating virus load. HIV-1 lymphoblastoid culture results are not closely related to the inoculum dose over several orders of magnitude (6). HIV-1 plasma culture techniques are subject to the same criticisms; in addition, their lack of sensitivity further compromises their routine use. Soluble HIV-1 p24 antigen measurements using serum, alcohol precipitated serum, or cerebrospinal fluid lack adequate sensitivity to detect viral products in more than 10-30% of all HIV-1 infected individuals (7). Direct analysis of HIV-1 DNA in blood mononuclear cells or tissues by Southern blot hybridization is capable of detecting viral sequences in only about 15% of cases (8); its limit of sensitivity of about 1 DNA sequence per 100 cells is inadequate for evaluation of HIV-1 infected materials. Direct analysis of cellular or free HIV-1 RNA sequences by hybridization methods also lacks adequate sensitivity for this purpose (9). In situ RNA hybridization is likely to be a highly sensitive and specific technique, but its technical difficulties make routine clinical use impossible (10).

The lack of adequate assays of virus load has seriously compromised the effectiveness of developing anti-viral therapies for HIV-1. HIV-1 cultures and p24 antigen measurements are used to monitor anti-viral efficacy in clinical trials (11). However, the lack of sensitivity and the biological variation in these measures make it difficult to rely on these data in assessing therapeutic efficacy. Thus, investigators must also rely on indirect immunological (e.g. T4 lymphocyte count, delayed hypersensitivity reaction) and clinical (e.g. development of opportunistic infections or neoplasms or death) criteria for monitoring such studies. A sensitive, specific, quantitative assay of HIV-1 virus load will signficantly increase the efficiency of evaluation of anti-viral agents, both in terms of time, cost, and patient morbidity and mortality.

A novel technique was developed in Dec., 1985 by Saiki and coworkers for amplification of DNA sequences (12). This technique is known as primer chain amplification reaction (PCR). DNA extracted from any tissue may be utilized in this assay. The two strands of DNA are denatured with heat. Specific oligonucleotides identical and complementary to sequences of 20-30 nucleotides on the 5′ and 3′ ends, respectively, of a DNA sequence of 100-2000 nucleotides are then annealed to the denatured DNA. A new strand of DNA is synthesized with a DNA polymerase. Thus after a single cycle of denaturation-annealing-polyerization, two copies of the sequence of interest are present. After two cycles, four copies are present, and after n cycles there are

$(1 + k)^n$ copies, where k is the efficiency of amplification at each cycle and ranges from 0 to 1. With a k value of 0.6, there are predicted to be 100,000 copies after 25 cycles. With a k value of 0.8, there are predicted to be about one million copies after 25 cycles.

Given the data from Southern blot hybridization directly from fresh tissues of HIV-1 infected individuals, we can estimate that on average there is about 1 DNA sequence per 300 cells, and that over 95% of individuals will have at least 1 DNA sequence per 1000 cells. The PCR technique should therefore allow amplification of rare HIV-1 sequences from undetectable to detectable levels. We have confirmed this prediction, as has several workers at Cetus in work published within the last year (13). However, the basic goal of quantitation of HIV-1 DNA sequences remains to be demonstrated and will be described further below.

Experimental Procedures and Results

We have used a variety of oligonucleotide primer pairs for amplification, including those with the 5′ long terminal repeat sequences (LTR), leader sequence, gag, or env genes. The positions of the oligonucleotides in regions other than env are shown in Fig. 1; oligonucleotides within env include SK68 and SK69 and have previously been described (13). These oligonucleotides were chosen by the following criteria:

a) Oligonucleotides of 17-24 nucleotides were chosen to insure annealing. The longer oligonucleotides were designed for experiments using the Thermus aquaticus (Taq I) polymerase in which case polymerization is carried out at 72 degrees C. Thus, stable annealing would be required at this temperature.

b) Oligonucleotides were designed to have a GC content of at least 50% to insure stable hybridization.

FIG. 1. Sequences of primers used for PCR. The nucleotide sequence of a portion of the 5' LTR, leader sequence, and *gag* gene is shown[15] with the nucleotide positions to the right of each line. The sequence of each primer and the positions of SacI restriction enzyme sites are indicated. The calculated T_m[16] for hybridization of each oligonucleotide is 58°C for P2, 68°C for P3, 84°C for P4, 62°C for P13, and 56°C for P14.

c) Oligonucleotides were chosen which flanked regions of 100-300 nucleotides. The efficiency of the amplification reaction declines significantly when amplifying larger regions of DNA.

d) Oligonucleotides were chosen which are identical or complementary to HIV-1 DNA sequences which are theoretically and/or empirically conserved among different HIV-1 isolates. This will increase the liklihood that a given set of primers will anneal to HIV-1 DNA sequences from a variety of different isolates. These regions include those surrounding the TATA box, the polyadenylation signal, the tRNA primer binding site, and the beginning of the gag gene which encodes a myristylated p17 protein.

e) Oligonucleotides were not made which are identical or complementary to regions of the HIV-1 genome which are likely to have homology with cellular sequences. Thus, sequences within pol which have homology to possible endogenous human retroviruses were not utilized.

In the initial work PCR was performed with E. coli DNA polymerase I Klenow fragment and the amplification cycles were performed by hand. Subsequent improvements in the technique which were incorporated later included the following:

a) Taq I polymerase became available in 11/87 and was substituted for Klenow polymerase at that time.

b) An automated PCR machine became available in 3/88 and was substituted for manual reactions at that time.

These improvements increased the speed of assays by a factor of about 50-fold and increased the efficiency of amplification.

In the initial experiments with Klenow polymerase, we demonstrated that with 20 cycles of amplification, using primers R13 and R14, cloned HIV-1 sequences could be amplified 10,000-fold. Thus, the efficiency per cycle is about 60%. Digestion of the cloned DNA with Sac I, which cuts between the two primer binding sites, reduced the efficiency of amplification dramatically. The size of the amplified product was 188 nucleotides, as predicted based on the sequences from this clone.

Using the same primer, amplification could be achieved with 1 microgram of HIV-1 infected H9 cell line DNA. In this case, amplification of more than 1000-fold was acheived as demonstrated by serial dilutions of the amplified product and slot blot hybridization analysis. The amplified product was 188 nucleotides in this case, as well. No HIV-1 DNA could be detected after amplification reactions using uninfected H9

cell DNA or MT2 DNA. The latter cell line is infected with HTLV-I but not HIV-1. HIV-1 DNA sequences could be detected after amplification using DNA derived from a peripheral blood mononuclear cell culture infected with a distinct isolate, JF.

In work with primers P2 and P3 and the Taq I polymerase, using 25 PCR cycles (denaturing at 94 degrees C for 2.5 min., annealing at 40 degrees C for 3 min., and polymerizing at 72 degees C for 2 min.) an amplified product of 223 nucleotides was obtained using 1 ng of cloned DNA. If a third primer, P4, was added, two different regions could be amplified simultaneously, yielding products of 129 and 223 nucleotides as predicted by available sequence data. However, if one adds a primer, R12, which is complementary to P3, amplification reactions are blocked, thus demonstrating the specificity of the technique.

Similar results were obtained with the Taq polymerase with cell line DNA as previously described in experiments utilizing the Klenow polymerase. Using primers P2 and P3, an amplified product of 223 nucleotides could be obtained from DNA of HIV-1 infected H9 cells, but not DNA from uninfected H9 cells.

The assay was then applied to DNA from fresh tissues of patients, in which the HIV-1 DNA sequence concentration is expected to be significantly lower. Tissues sources included samples from peripheral blood mononuclear cells, brain, lymph node, or spleen. Using primers P2 and P3, and the Taq polymerase, an amplified product of 223 nucleotides was obtained in tissues from 10 of 12 HIV-1 infected patients. HIV-1 DNA sequences could be detected in the amplified products of samples from all types of tissues described above, including samples that were negative by direct Southern blot hybridization analysis. The PCR assay performed with a single set of primers failed to amplify HIV-1 DNA sequences from two different brain samples of one HIV-1 infected patient or from lymph node and spleen samples from a second HIV-1 infected patient. No amplified HIV-1 DNA sequences were detected in any of the nine HIV-1 negative patients evaluated, demonstrating the specificity of the reaction.

Under the reaction conditions described above, a similar quantity of HIV-1 DNA sequences was present in the amplified DNA from all sources, including cloned DNA, infected cell line DNA, or fresh patient material. It is expected that the reactions were saturating, and the concentration of the final reaction products was not a reflection of the initial HIV-1 sequence concentration. In the first attempt to quantitate HIV-1 DNA sequences, we performed amplification with a variety of concentrations of cloned DNA ranging from 1 ng to 1 fg over differing numbers of cycles of amplification, 10, 15, or 20 cycles. Under these conditions, the concentration of HIV-1 sequences in the amplified products are proportional

to the initial HIV-1 DNA sequence concentrations, thus demonstrating the ability of the assay to quantitate HIV-1 DNA sequences.

In addition, we have utilized the PCR technique for the detection of HTLV-I and HTLV-II sequences from fresh patient material (Fig. 2). Oligonucleotides were synthesized which were identical to nucleotides 7463-7486 and complementary to nucleotides 7552-7572 in the rex and tax genes of HTLV-I (14). Identical sequences are present in the HTLV-II genome (15). Amplification was performed with Taq polymerase for 30 cycles under the conditions described above using either cloned HTLV-I or HTLV-II sequences, HTLV-I infected cell lines MT2 and HUT 102, or peripheral blood or lymph node material from 6 HTLV-I infected individuals that have been referred to us. Three of these individuals had acute adult T cell leukemia/lymphoma (ATLL), one had chronic ATLL, one had an intermediate clinical syndrome, and one was asymptomatic. HTLV sequences could be detected in all samples but not from the negative controls which included the chronic myelogenous leukemia cell line K562 or the uninfected T lymphoid cell line H9. HTLV-I or HTLV-II-specific endlabeled oligonucleotide probes were utilized to distinguish in each case which HTLV sequences were present.

We have found that the use of acid citrate dextrose (ACD) tubes for collection of blood for PCR studies rather than heparin to be advantageous for several reasons. First, the yield of undegraded DNA is significantly higher using ACD as a preservative, especially when blood samples are not processed for 2-3 days. Second, we have found that heparin contaminants can inhibit PCR reactions.

We have succeeded in quantitating HIV-1 DNA sequences from fresh tissues by comparing the level of HIV-1 sequences to those of globin (Fig. 3).

Figure 2 is an ethidium bromide stained agarose gel of PCR products of HIV-1 env or human heparin co-factor II gene (HCII) sequences. The lack of a signal in lanes A1 and A16 and B1 and B16 from PCR reactions performed with no DNA demonstrates the specificity of the PCR reactions. Furthermore, no HIV-1 PCR product is identified with uninfected H9 DNA (A2) nor is a HCII PCR product identified with murine DNA (B2). The relationship of the amount of PCR product to starting DNA concentrations is shown in lanes A3-A7 and B3-B7. Starting with 1000 or 100 fg of HIV-1 DNA (lanes A3 and A4) (equivalent to 1 HIV-1 genome per 3 or 30 cells, respectively) saturating amounts of PCR product are obtained. Lower levels of PCR products are obtained when one starts with 10, 1, or 0.1 fg (lanes A4-A6) of HIV-1 DNA (equivalent to 1 HIV genome per 300, 3000, or 30,000 cells, respectively). No significant difference in the concentration of PCR products is apparent in this concentration range. With HCII DNA, after 40 cycles of PCR, a product is apparent only if one starts with 1000 fg of cloned DNA

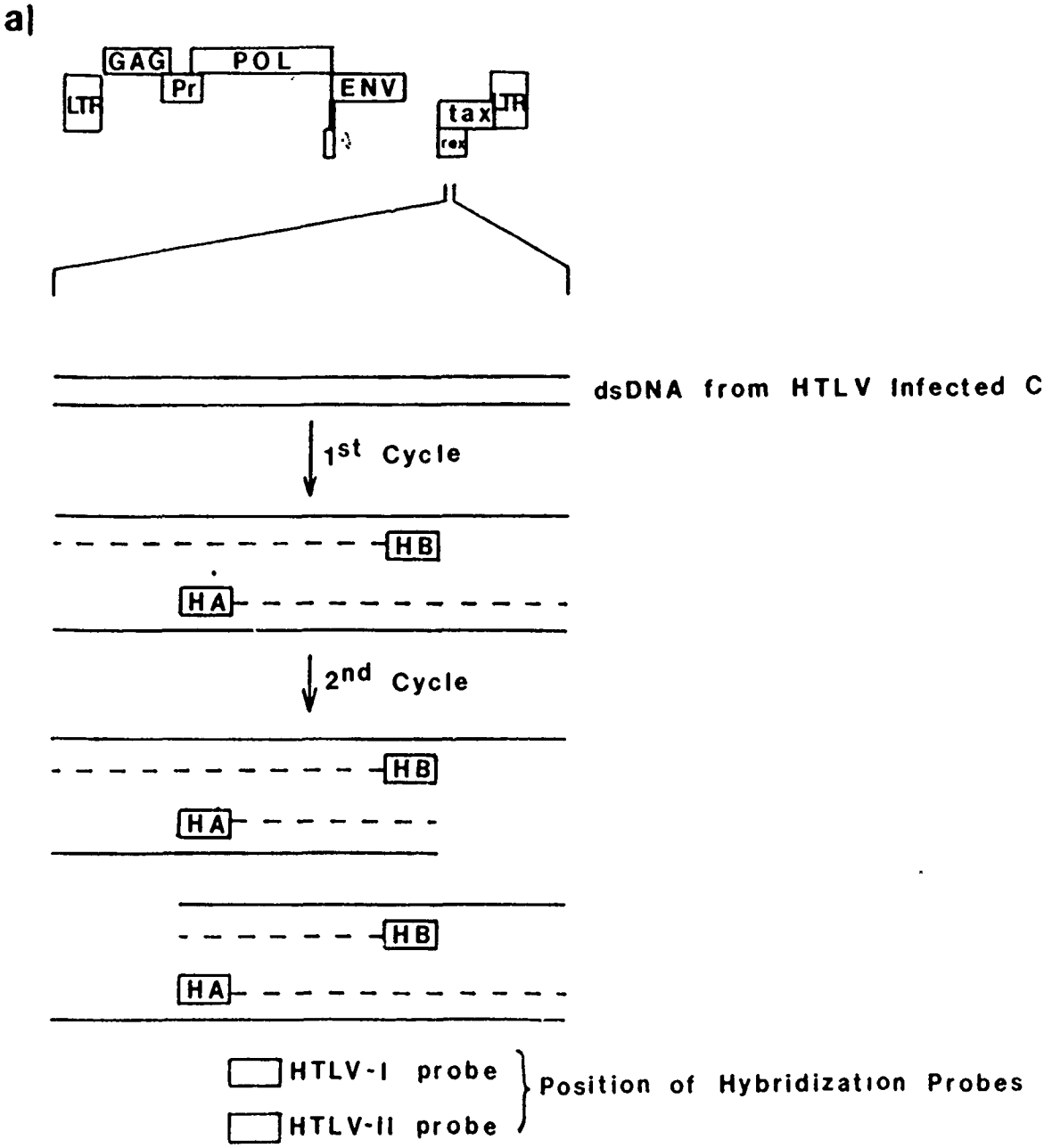


FIG. 2 Detection of HTLV-I DNA sequences by polymerase chain amplification assay using fresh tissues from patients with ATLL. A. A schematic map of the HTLV-I genome is shown with the relative position of each gene. The region amplified with oligonucleotides HA and HB is found within the rex and tax genes. A schematic for 2 cycles of the amplification reaction is shown. During each cycle, DNA is denatured, annealed to the oligonucleotide primers, and a new strand of DNA is synthesized (dotted lines). The concentration of DNA sequences between the 2 primers increases exponentially with the number of cycles of amplification. The positions of the HTLV-I and -II hybridization probes relative to the amplified segment of DNA are indicated. B. DNA from peripheral blood mononuclear (PBM) or bone marrow cells (BM), uninfected K562 or H9, or the HTLV-I infected HUT 102 cell line, or cloned HTLV-I or -II DNA sequences were used for amplification. Polymerase chain amplification reactions were performed by the method of Saiki and colleagues (171) using the Thermus aquaticus polymerase (Cetus). 0.5 micrograms of DNA was used for each sample. Primers are identical and complementary to HTLV-I nucleotides 7463-7486

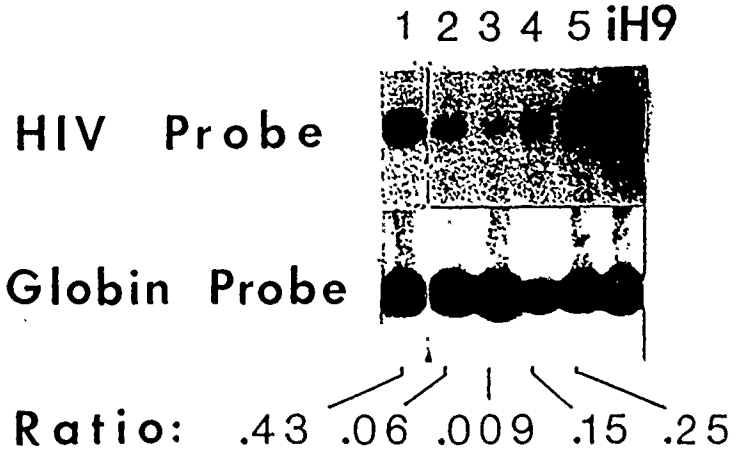


Fig. 3. PCR AMplification of HIV or globin sequences from fresh peripheral blood mononuclear cell DNA. HIV-1 sequences were amplified with primers P2 and R16 and globin sequences with RS05 and RS06. Samples were electrophoresed on an agarose gel and Southern blot hybridized with specific probes. The ratio of HIV to globin densitometric signals is listed at the bottom, demonstrating the wide ragge of variation of HIV sequences in vivo.

(B3). With the patient samples, HCII PCR products can be identified if one starts with 1.0 or 0.1 micrograms of DNA (B8, B9, B13, B14), or 0.01 micrograms in the case of one patient (B14), but not with 0.001 micrograms of DNA (B11, B15). HIV PCR products could be detected from 1, 0.1, or 0.01 micrograms of DNA from pt 464 (A8, A9, A10), but only from 0.1 micrograms of DNA from pat. 118 (A14). HIV PCR analysis with 1 microgram of pt 118 DNA could not be evaluated due to a smear (A13).

The Table summarizes the results of analyses performed in duplicate or triplicate on DNA samples from 33 patients. Ethidium bromide stained gels of PCR products of HIV-1 leader or env sequences or control HCII sequences are listed as intense (++), detectable (+), or undetectable (-). In 12-27% of PCR reactions, evaluation for the presence or absence of PCR products was not possible due to a smear. This likely represents amplification of non-specific PCR products and/or degradation of specific PCR products. The ratio of HIV to control products was estimated as high, intermediate, or low in each case. In 27 cases (12 with HIV-1 leader sequences and 15 with HIV-1 env sequences), HIV DNA concentrations could not be estimated due to inevaluable HIV PCR reaction products, or undetectable or inevaluable HCII reaction products. In 18 of 33 cases, HIV DNA concentrations could be estimated for both HIV-1 leader and HIV-1 env sequences. In 13 cases, concordant levels of HIV-1 leader and env sequences were found. In all discordant cases, HIV-1 env DNA concentrations were lower than HIV-1 leader DNA concentrations.

In addition to agarose gel and ethidium bromide staining of PCR products, dot blot hybridization was also performed. Examples of these experimental results are shown in Fig. 4 and 5. In dots A1, B1, and C1, in each figure, no DNA was included. In Fig. 3 C1 and Fig. 4 B1 and C1, signals are noted, suggesting difficulties with "carry-over" of DNA. In A2, B2, and C2, reaction products with negative controls with uninfected human (H9) DNA (A2 and B2) or murine (3T3) DNA (C2) are shown. The positive signals in Fig. 4, B2 and C2 and Fig. 5, A2 and B2 also suggest problems with "carry-over."

In dots A3-A7 and B3-B7, decreasing amounts (1000, 100, 10, 1, or 0.1 fg) of cloned HIV-1 sequences were added to PCR reactions with H9 DNA. Generally, the amount of PCR products decreased with decreasing amounts of HIV-1 DNA sequences. However, exceptions are noted in which spuriously large amounts of PCR products (Fig. 4, A7 and B7, Fig. 5 B7) or low amounts (Fig. 5 B3) were obtained. Similar results were obtained with decreasing amounts of cloned HCII DNA sequences (C3-C7).

Table

Analysis of Ethidium Bromide Stained PCR Products of HIV-1 Infected Individuals

Patient	Disease Stage (CDC)	Leader	Env	Control	Leader	Env
014	IVA	-	-	-	NE	NE
049	III	+	NE	-	High	NE
066	IVA	++	++	++	Int	Int
069	IVC2	-	-	-	NE	NE
073	IVC2	++	-	+	High	None
113	III	++	-	++	Int	None
114	III	-	NE	NE	NE	NE
116	IVA	++	++	++	Int	Int
117	III	+	NE	++	Low	NE
118	III	++	-	++	Int	None
121	IVC2	+	+	++	Low	Low
133	III	++	++	++	Int	Int
134	III	-	-	-	NE	NE
150	II	-	-	-	NE	NE
205	II	++	++	++	Int	Int
207	III	++	++	+	High	High
225	III	++	++	++	Int	Int
281	IIII	-	-	-	NE	NE
382	IVC2	NE	NE	NE	NE	NE
449	II	++	+	++	Int	Low
453	IVA3	+	+	+	Int	Int
459	III	+	-	+	Int	None
464	II	++	NE	NE	NE	NE
467	II	NE	NE	NE	NE	NE
469	IV	+	+	+	Int	Int
473	III	++	NE	+	High	NE
477	III	-	-	+	None	None
488	II	NE	NE	NE	NE	NE
489	O	NE	NE	NE	NE	NE
523	III	++	++	++	Int	Int
524	III	++	++	++	Int	Int
527	IVA	++	++	++	Int	Int
531	IVC1	-	-	-	NE	NE

++ intense band
+ detectable band
- no band
NE not evaluable (smear) or undetectable control
High stronger signal with HIV than control
Int similar signal with HIV as control
Low weaker signal with HIV than control
None no signal with HIV, but signal with control

All assays performed in duplicate or triplicate, with very little variation using 1.0 microgram of DNA.

HIV-1 DNA PCR DATA AT DIFFERENT DISEASE STAGES

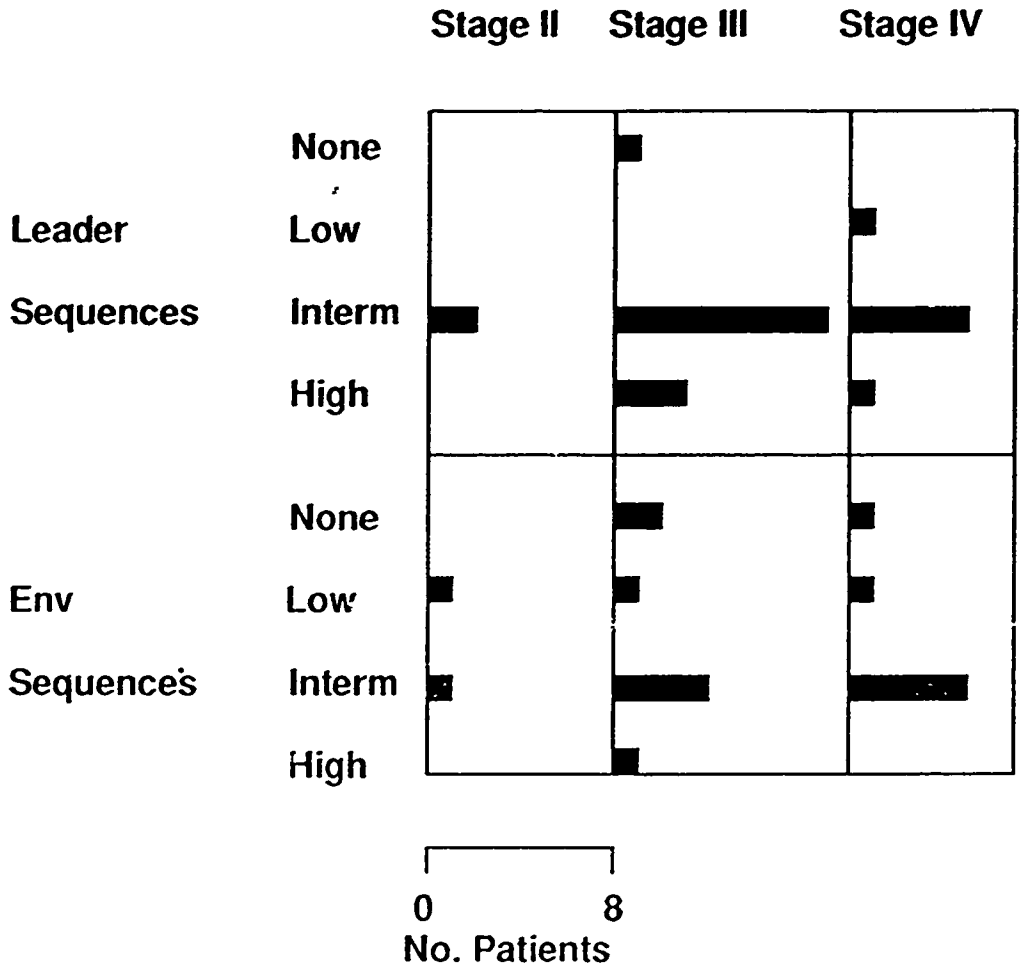


Table 1

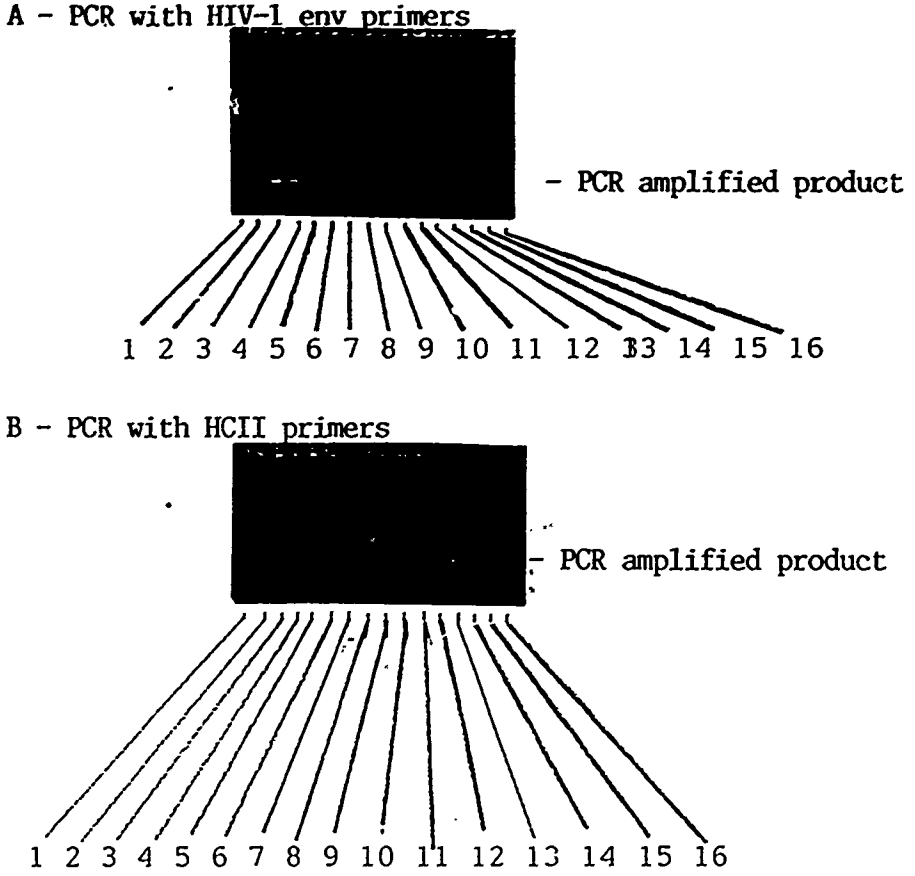
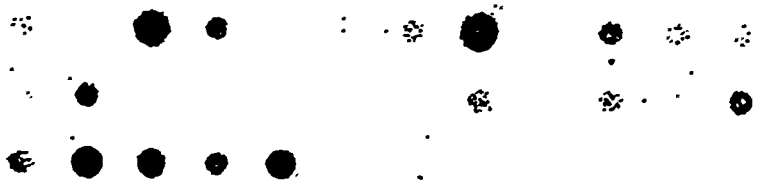


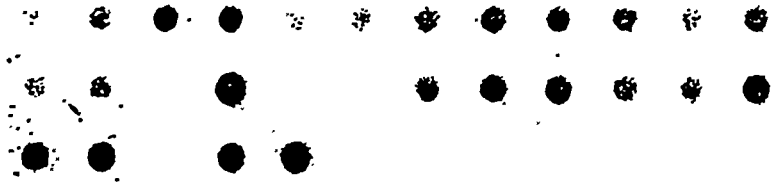
Fig. 3. Agarose gel analysis of HIV-1 and heparin co-factor II products. In each case, no DNA was included in lanes 1 and 16. Uninfected H9 DNA (A2) or 3T3 DNA (B2) only was included in lanes 2. In lanes 3-7, 1000, 100, 10, 1, or 0.1 fg of cloned HIV-1 (A) and HCII (B) DNA was added. In lanes 8-11, 1000, 100, 10, or 1 ng of DNA from peripheral blood mononuclear cells of patient 464 was used for PCR. In lanes 12-15, the products from PCR analysis with 1000, 100, 10, or 1 ng of DNA from peripheral blood mononuclear cells from patient 118 are shown.

primers for HIV-1 env sequences:	E2A	GAGGATATAATCAGTTTATGGGATCAAA
	E3B	ATTCCATGTGTACATTGTACTGT
primers for HCII sequences	oligodT	GGGAGCAAAGGCCCGCTGGATCAGCTAGAG
	oligo 21	TTTAGTTCTGAAGTCAAGCAG

A - PCR products of HIV-1 leader



B - PCR products of HIV-1 env



C - PCR products of HCII sequences

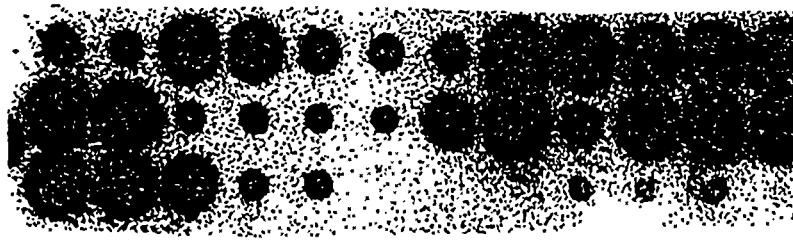


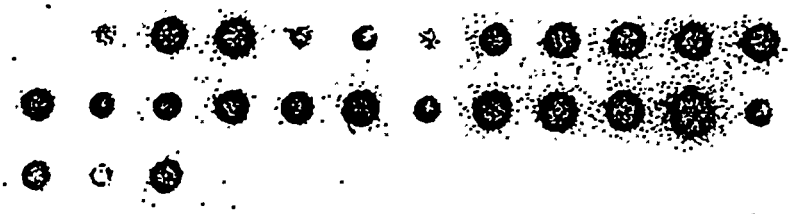
Fig. 4. Autoradiograph of Dot Blot Hybridication of PCR Products. PCR reactions were performed with (1) no DNA, (A2,B2) H9 or (C2) 3T3 DNA only or the same DNA supplemented with (3) 1000, (4) 100, (5) 10, (6) 1, or (6) 0.1 fg of cloned (A,B) HIV-1 or (C) HCII DNA, or 1 microgram of peripheral blood mononuclear cell DNA from patient number (8,9) 464, (10,11) 133, (12,13) 118, (14,15) 459, (16,17) 113, (18,19) 230, (20,21) 003, (22,23) 479, (24,25)084, and (26,27) 166. Primers for HIV-1 leader sequences are:

P2 AGCTGCTTTTTGCCTGTACT
R16 GACGCTCTCGCACCCATCTCTCTC

Primers for HIV-1 env and HCII sequences are listed in Fig. 2 legend. 40 cycles of PCR were performed.

A - PCR products with HIV-1 leader

Sample Nos.

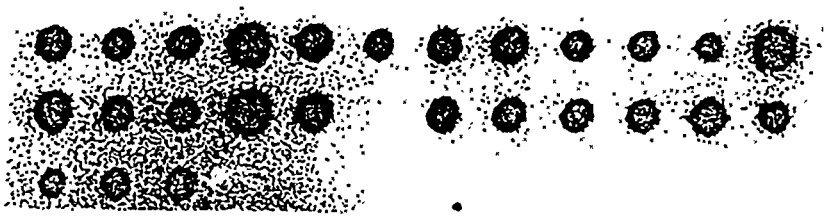


1-12

13-24

25-27

B - PCR products with HIV-1 env

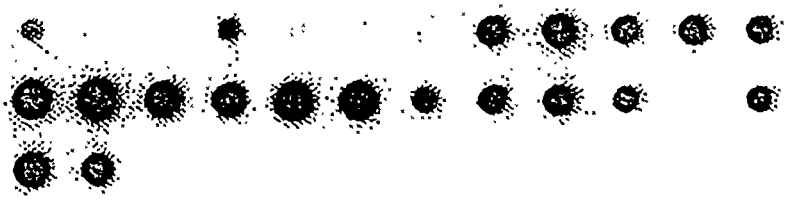


1-12

13-24

25-27

C - PCR products with HCII sequences



1-12

13-24

25-27

Fig. 5. Autoradiograph of Dot Blot Hybridization of PCR Products. PCR reactions were performed with (1) no DNA, (A2, B2) H9 or (C2) 3T3 DNA only or the same DNA supplemented with (3) 1000, (4) 100, (5) 10, (6) 1, or (7) 0.1 fg of cloned (A,B) HIV-1 or (C) HCII DNA, or 1 microgram of peripheral blood mononuclear cell DNA from patient (8) 477, (9) 212, (10) 069, (11) 469, (12) 049, (13) 473, (14) 225, (15,16) 489, (17) 488, (18) 382, (19) 467, (20) 464, (21) 133, (22) 118, (23) 459, (24) 113, (25) 230, (26) 003, (27) 479, (28) 084, and (29) 166. Primers and PCR reactions were performed as described in Fig. 3 legend.

The remainder of the reaction products were obtained from patient DNA samples. The results shown in Fig. 3 are from duplicate patient samples, and show good concordance. However, divergent results are noted in a few duplicate samples (Fig. 4 B15 and B16, Fig. 5 A18 and A19 and B18 and B19).

The results in Fig. 4 and 5 can not be compared with one another due to differences in the lengths of exposure of the autoradiograms. The most notable finding in comparing the data in these figures to the data from agarose gel analysis is the more limited variation in concentration of HIV sequences as estimated by hybridization analysis compared to agarose gel and ethidium bromide staining.

Conclusions

The experiments performed in this study have demonstrated a number of important findings.

1) Low concentrations of HIV-1 DNA sequences can be amplified from levels which are undetectable by Southern blot hybridization to levels which can be easily detectable.

2) The PCR reactions can be successfully performed with either E. coli DNA polymerase I or Taq polymerase. The latter DNA polymerase is advantageous since it is not inactivated during repetitive cycles of heat denaturation. Thus, using this polymerase no additions to the reaction are needed after each cycle.

3) The PCR reactions are greatly facilitated by an automated machine. This speeds up the reaction considerably, and also provides for more efficient amplification.

4) Using Klenow polymerase, we calculated the efficiency of amplification as 60% per cycle with cloned DNA. A similar amplification efficiency was estimated with the Taq polymerase.

5) The specificity of the amplification with cloned DNA was demonstrated by two different methods. Amplification was dramatically decreased by a) digestion of the target DNA with an enzyme which cuts between the primer binding sites, or b) the addition of a blocking oligonucleotide complementary to one of the two oligonucleotides used for amplification.

6) Small amounts of HIV-1 DNA sequences in cell lines infected with either of two HIV-1 isolates could be detected after PCR, whereas they could not detected in 1 microgram samples by direct Southern blot hybridization. The amplification efficiency over 20 cycles with infected cell line DNA

is greater than 1000-fold. No signal was obtained using DNA from cell lines not infected with HIV-1, including HTLV-I infected cell lines.

7) Using a single set of primers, amplification could be obtained with tissue samples from 10 of 12 patients. The technique was successful with a wide range of different tissue types including brain, blood mononuclear cells, lymph node, and spleen. The technique was successful with samples in which HIV-1 DNA sequences could not be detected directly by Southern blot hybridization on the unamplified DNA. Thus, with a single set of primers, a sensitivity of over 90% was acheived. No amplified HIV-1 sequences were detected in two different tissues from each of two HIV-1 infected individuals with symptomatic disease. It is likely that in these cases, sequence heterogeneity at the primer binding sites accounted for the failure of the PCR reactions. With the use of additional primers to other conserved regions of the HIV-1 genome, it is likely that HIV-1 DNA sequences could be amplified in these samples as well.

8) HIV-1 DNA sequences could not be detected after PCR under identical conditions from any of the nine samples from uninfected individuals. This demonstrates the specificity of the method.

9) PCR allows quantitation of HIV-1 DNA sequences when performed under non-saturating reaction conditions. This was demonstrated with differing amounts of cloned HIV-1 DNA ranging from 1 ng to 1 fg of DNA, and differing number of PCR cycles, 10, 15, or 20 cycles.

10) PCR allows quantitation of HIV-1 DNA sequences from peripheral blood mononuclear cells. However, inconsistent results are occasionally obtained when examining results of duplicate analyses or concentration curves. This is likely due to marked amplifications (>one million-fold) of small amounts of DNA. In fact, if a single DNA molecule is present in a sample, Poisson distribution theory would predict positive results in 66% of cases and negative results in 34% of cases. Agarose gel analysis and ethidium bromide staining or Southern blot hybridization of PCR products demonstrates significant variation of PCR product results from different patients. This is consistent with the presence of a wide range of levels of virus in vivo. However, the level of HIV-1 DNA sequences in vivo did not correlate with disease stage. It should be noted that this assay measures the total number of HIV-1 DNA sequences per mononuclear cell in the blood. It does not measure any of the following:

a) Number of HIV-1 DNA sequences per CD4+ cell
b) Number of HIV-1 DNA sequences per infected cell
c) Number of HIV-1 DNA sequences in tissue samples other than blood
d) Number of HIV-1 DNA sequences which are actively expressed

Any of these latter measurements may correlate better with disease stage.

Very little variation is noted in results analyzed by dot blot hybridization. This result may be due to:

a) partial binding of oligonuceotides from PCR reactions to nitrocellulose filters and labeled probes

b) failure to use a molar excess of labeled probe in dot blot hybridization analyses.

11) Amplification of HTLV-I and II sequences was successful using primers to sequences which are identical in the rex/tax genes of both HTLV-I and II. HTLV sequences could be detected in fresh tissues from symptomatic and asymptomatic patients, utilizing DNA samples which are either positive or negative by Southern blot hybridization. Thus, this provides a highly sensitive method of diagnosing HTLV infection of either type. It may be useful as a confirmatory assay or a primary screening assay. This will be increasingly important with the development of screening assays for these and other retroviruses in blood banks and particular patient populations. HTLV-I and II could also be distinguished by hybridizing these amplified products with HTLV-I or II-specific oligonucleotides. Since most antibody-based assays are unable to make this distinction, this is an important feature of this new assay which should assist in the diagnosis of HTLV infections.

Recommendations

The work outlined in this report should be continued in two ways:

1) Quantitation of HIV-1 DNA sequences will be pursued with funding from the Washington University AIDS Clinical Trials Unit (AI25903).

For this purpose, agarose gel analysis is preferable to dot blot hybridization analysis. Southern blot analysis has also been useful, but is more time consuming, and more tedious to perform, and will prove difficult for a clinical laboratory. Difficulty in estimating DNA concentrations by agarose gel analysis should be overcome by the use of serial concentrations of input DNA. Therefore, for further analysis, we will use 1.0, 0.1, 0.01, and 0.001 microgram quantities of DNA.

We will also calculate the HIV-1 DNA sequence level per CD4+ cell.

2) HIV-1 RNA PCR analysis should be performed with continued funding from DAMD when available. This work will focus on vif, tat, rev, and nef RNAs to correlate their presence of amounts with clinical stage.

3) HTLV-I and II DNA PCR should be applied to distinguish these viruses. This distinction has epidemiological and clinical importance.

Publications

Three manuscripts from this work have been published, two manuscripts are in press, two manuscript have been submitted for publication, and two manuscript are currently being prepared for submission.

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